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Integration of growth factor signals at the c-fos serum response element

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SUMMARY

A transcription factor ternary complex composed of serum response factor (SRF) and a second factor, ternary complex factor (TCF), mediates the response of the c-fos Serum Response Element to growth factors and mitogens. In NIH3T3 fibroblasts, TCF binding is required for transcriptional activation by the SRE in response to activation of the Ras-Raf-ERK pathway. We compared the properties of three members of the TCF family, Elk-1, SAP-1 and SAP-2 (ERP/NET). Although all the proteins contain sequences required for ternary complex formation with SRF, only Elk-1 and SAP-1 appear to interact with the c-fos SRE efficiently *in vivo*. Each TCF contains a C-terminal activation domain capable of transcriptional activation in response to activation of the Ras-Raf-ERK pathway, and this is dependent on the integrity of S/T-P motifs conserved between all the TCF family members. In contrast, activation of the SRE by whole serum and the mitogenic phospholipid LPA requires SRF binding alone. Constitutively activated members of the Rho subfamily of Ras-like GTPases are also capable of inducing activation of the SRE in the absence of TCF; unlike activated Ras itself, these proteins do not activate the TCFs in NIH3T3 cells. At the SRE, SRF- and TCF-linked signalling pathways act synergistically to potentiate transcription.

1. INTRODUCTION

The Serum Response Element (SRE) is a regulatory sequence found in many growth-factor and mitogen regulated promoters (reviewed by Treisman 1990). The c-fos SRE binds a transcription factor ternary complex comprising the transcription factors SRF (Serum Response Factor; Norman *et al.* 1988) and TCF (Ternary Complex Factor; Shaw *et al.* 1989). TCF binds an Ets motif adjacent to the SRF binding site, but cannot do so independently of SRF; genomic footprinting studies show that the ternary complex is present at the SRE even in unstimulated cells, suggesting that SRE activation involves regulation of transcriptional activation rather than DNA binding (Herrera *et al.* 1989). TCF binding is required for efficient response of the c-fos promoter to activators of the Ras-Raf-ERK signalling pathway such as v-raf and phorbol ester (Graham & Gilman 1991; Hill *et al.* 1994; Kortenjann *et al.* 1994).

Elk-1, SAP-1, and ERP/NET are three Ets domain proteins with DNA binding properties characteristic of the TCFs; they contain three regions of substantial sequence homology termed boxes A, B and C (see figure 1*a*; Hipskind *et al.* 1991; Dalton & Treisman 1992; Giovane *et al.* 1994; Lopez *et al.* 1994). Boxes A and B mediate DNA binding and cooperative interaction with SRF at the SRE (for review see (Treisman 1994; also Shore & Sharrocks 1994). The C-terminal region, including box C, contains several conserved potential MAP kinase phosphorylation sites, and is a substrate for ERK phosphorylation *in vitro* (Janknecht *et al.* 1993; Marais *et al.* 1993; Gille *et al.* 1995*a*). Phosphorylation of Elk-1 potentiates transcriptional activation, and under certain conditions, its DNA binding activity (Hill *et al.* 1993; Janknecht *et al.* 1993; Marais *et al.* 1993; Kortenjann *et al.* 1994). The regulatory properties of the other TCF proteins have remained less well defined.

While it is well established that binding of TCF to the c-fos SRE links the c-fos promoter to the Ras-Raf-ERK pathway, c-fos promoter mutants that cannot bind TCF remain responsive to signals elicited by whole serum, the mitogenic phospholipid LPA, or global activation of heterotrimeric G proteins (Graham & Gilman 1991; Hill *et al.* 1994; Hill & Treisman 1995*b*). Serum-induced TCF-independent activation of the SRE requires SRF (Hill *et al.* 1994; Johansen & Prywes 1994). SRF must bind DNA via its own DNA binding domain to regulate transcription, and activation is blocked by DNA binding domain mutations which do not impair DNA binding (Hill *et al.* 1994). The results suggest that SRF adopts an 'active' conformation upon binding DNA which allows it to interact with a non-TCF accessory factor activated by serum-induced signals (Hill *et al.* 1994).

Here we investigate the interactions between different cellular signalling pathways at the c-fos SRE. We show that both SAP-1 and Elk-1, but not SAP-2, interact efficiently with SRF *in vitro* and *in vivo*, and that all three proteins respond to activation of the Ras-Raf-ERK pathway. In contrast, SRF responds weakly

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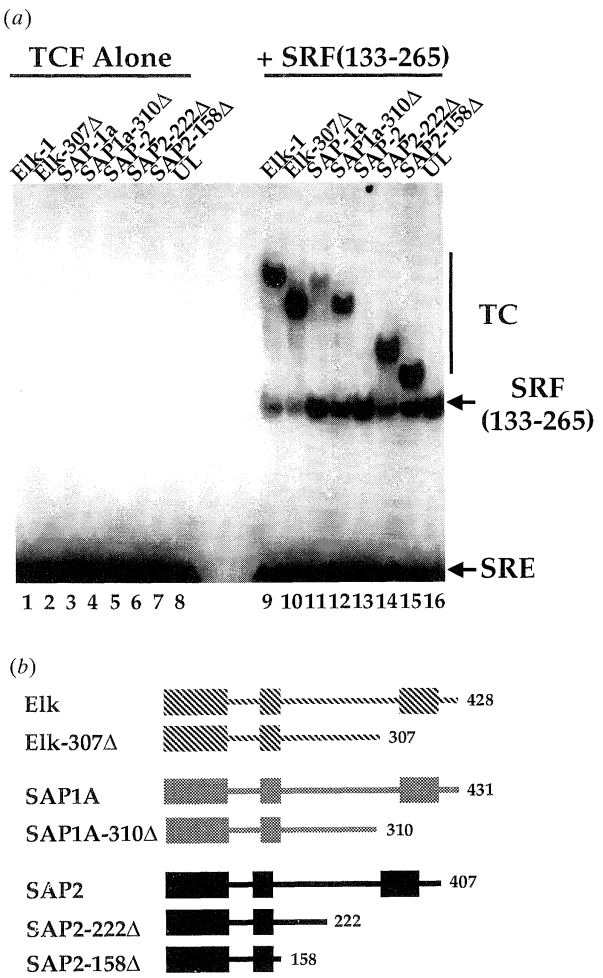

Figure 1. Structure and *in vitro* binding by TCF proteins. Ternary complex formation *in vitro* by the three TCF proteins. Gel mobility shift assays were performed with a c-fos SRE probe in the absence (lanes 1–8) or presence (lanes 9–16) of SRF(133–265), using TCF derivatives produced by *in vitro* translation. UL, unprogrammed reticulocyte lysate. Ternary complexes, the SRF(133–265):SRE complex, and free probe are indicated. The structure of the TCF derivatives is shown below, with the homology regions (Dalton & Treisman 1992) shown as boxes.

to activation of the Ras-Raf-ERK pathway but is strongly activated by expression of activated forms of members of the Rho family of Ras-related GTPases. Maximal transcriptional activation by the SRE requires that both Rho- and Ras-linked pathways be simultaneously activated.

2. MATERIALS AND METHODS

(*a*) *Plasmids*

Expression plasmids encoding the Elk-1, SAP-1, and SAP-2 derivatives, or activated signalling molecules, have been described (Hill et al. 1993; Marais *et al.* 1993; Hill *et al.* 1994; Hill & Treisman 1995a; Price *et al.* 1995). Reporter plasmids contain the CAT gene linked to the HSV thymidine kinase promoter, controlled by two copies of either the wildtype c-fos SRE (SRE.tkCAT), or mutant SRE derivatives containing a LexA halfoperator in place of the Ets motif (SRE.L.tkCAT) or an MCM1 site in place of the SRF site (SRE.M.tkCAT). For LexA-TCF fusion protein studies a reporter conrolled by a dimeric LexA operator (2LexOP.tkCAT) was used (Hill *et al.* 1993; Marais *et al.* 1993; Hill *et al.* 1994).

(*b*) *Cell Culture, Transfections and extract preparation*

Maintenance and DEAE-dextran transfection of NIH 3T3 cells, and preparation of extracts for CAT assays were as described (Hill et al. 1993; Marais *et al.* 1993; Hill *et al.* 1994). In general activity of a cotranfected lacZ reference plasmid varied less than 2-fold between any transfected sample. CAT activity was determined by standard methods. For study of regulated SRE activity, activity of the wildtype SRE reporter stimulated with 5% serum was taken as 100.

(*c*) *Gel mobility-shift assays*

TCF derivatives were produced by cell-free translation (Promega TNT) and quantitated by ^{35}S methionine incorporation, measured using the Phosphorimager. Gel mobility-shift assays for the ternary complex were as previously described (Marais *et al.* 1993).

3. RESULTS

(*a*) *DNA binding and ternary complex formation by SAP-2* in vitro

Three TCF cDNAs have been isolated, including Elk-1 (Hipskind *et al.* 1991), SAP-1 (Dalton & Treisman 1992) and SAP-2 (Price *et al.* 1995; a human homolog of the mouse ERP/NET gene (Giovane *et al.* 1994; Lopez *et al.* 1994)). Each contains the three characteristic conserved regions shared by the Elk-1 and SAP-1 TCFs (Dalton & Treisman 1992); their RNAs are present at similar relative levels in many different tissues. To compare the ability of the TCFs to form ternary complexes at the c-fos SRE, we produced the intact proteins or truncation derivatives by cell free translation and analysed complex formation at the c-fos SRE using the gel mobility-shift assay. None of the three proteins bound the SRE autonomously (figure 1, lanes 1,3,5). In the presence of SRF(133–265), an SRF fragment sufficient for ternary complex formation, Elk-1 and SAP-1A formed ternary complexes but SAP-2 did not (figure 1, compare lanes 9,11,13). C-terminal truncation of each TCF increased its DNA binding or ternary complex factor activity; indeed, ternary complex formation by SAP-2 truncations was comparable to that of analogous derivatives of the other proteins (figure 1; lanes 10,12,14,15). Analysis of further deletion derivatives of SAP-2 showed that sequences both within the C box and N-terminal to it contributed to inhibtion of ternary complex formation by SAP-2, and that the inhibitory effect is not dependent on specific SAP-2 N-terminal sequences (data not shown).

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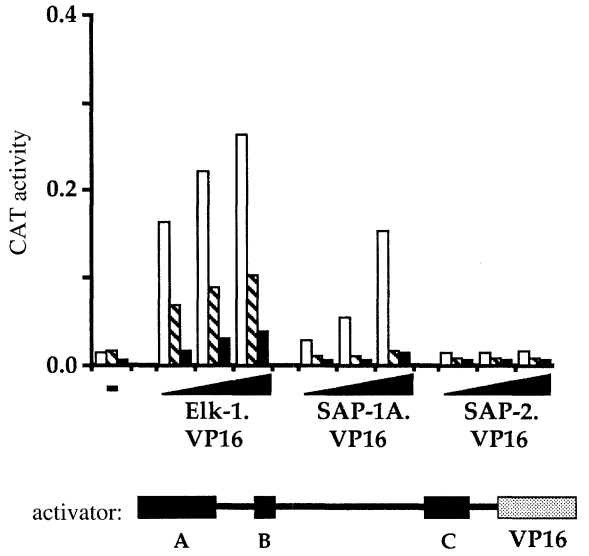

Figure 2. Elk-1 and SAP-2 but not SAP-2 can bind the c-fos SRE *in vivo*. NIH3T3 cells were transfected with SRE reporter plasmids together with VP16-tagged derivatives of the Elk-1, SAP-1 and SAP-2 as shown below. CAT activity in serum-deprived cells was determined after 48hr. Reporter plasmids contained either the wildtype c-fos SRE (WT SRE; open bars); SRE.L, a c-fos SRE with a mutated ets site (SRE.L; hatched bars); or SRE.M, a c-fos SRE with a mutated SRF site (SRE.M, solid bars). Transfections containing increasing amounts of expression vector are indicated by the solid triangles. A representative experiment is shown.

(*b*) *Elk-1 and SAP-1, but not SAP-2, can bind the c-fos SRE in serum-starved cells*

We next tested whether the three TCF proteins could bind the c-fos SRE *in vivo*. Fusion proteins containing the SAP-1 or Elk-1 A and B boxes linked to the constitutively active transcriptional activation domain from the HSV VP16 protein can activate an SRE-controlled reporter gene, but only if the SRE contains an intact SRF binding site: transcriptional activation thus reflects ternary complex formation *in vivo* (Dalton & Treisman 1992; Hill *et al.* 1994). We constructed fusion proteins comprising the entire TCF coding regions N-terminal to the VP16 activation domain and used the assay to test their ability to bind form ternary complexes at the c-fos SRE *in vivo*.

The VP16-tagged Elk-1 and SAP-1A derivatives efficiently activated the wild-type c-fos SRE (WT SRE) in serum-deprived NIH3T3 cells; activation was dependent both on the integrity both of the SRF binding site and the Ets motif contacted by the TCF Ets domain (figure 2; SRE.L, no Ets motif; SRE.M, no SRF binding site). In contrast, activation by the SAP-2 fusion proteins was barely above background levels, even though control immunoblotting experiments showed that expression of SAP-2/VP16 was comparable to that of Elk-1/VP16 and several-fold greater than expression of SAP-1A/VP16 (figure 2; data not shown). Thus, in the absence of growth factor stimulation it appears that Elk-1 and SAP-1A, but not SAP-2, can efficiently bind the c-fos SRE. We were also unable to detect significant SAP-2 ternary complex activity in extracts of cells stimulated by serum or TPA, or expressing activated Ras protein (see Discussion; Price *et al.* 1995).

(*c*) *Transcriptional Activation by the TCF C-termini*

We previously showed that a fusion protein composed of the bacterial LexA repressor linked to the Elk-1 C-terminal region can confer serum-regulated transcriptional activation on a LexA operator-controlled reporter gene, which alone is unable to respond to serum stimulation (figure 3*a*; Marais *et al.* 1993). Although serum stimulation also increases the ability of the fusion protein to bind the LexA operator, the failure of the mutant fusion proteins to activate transcription is not caused by defective DNA binding since point mutants that abolish activation do not abolish DNA binding (data not shown; see Price *et al.* 1995). To compare the ability of the TCFs to regulate transcription, we constructed analogous LexA fusion proteins containing wildtype or mutant derivatives of the SAP-1A and SAP-2 C termini (figure 3*a*). In the presence of the SAP-1A fusion protein NLex.SAP-1C, regulated activity of the reporter gene in response to serum, phorbol esters, and CSF1 stimulation was comparable to that brought about by NLexElkC (figure 3*a*; data not shown).

Serum-regulated transcriptional activation by NLex.ElkC is dependent on the integrity of several conserved C-terminal S-T/P phosphoacceptor sites; of these, serine 383 is especially important (Marais *et al.* 1993). We therefore tested the effects of analogous mutations in the NLex.SAP-1C fusion protein (figure 3*a*). Mutation to alanine of SAP-1A serine 381 (corresponding to Elk-1 residue 383) severely reduced transcriptional activation, although this mutation was less detrimental than the analogous Elk-1 mutation. Substitution of SAP-1A residues S387, T420 and T425 by alanine also reduced activation, but less effectively; a S381A/S387A double mutant was almost completely inactive, similar to its Elk-1 counterpart (figure 3*b*). These data show that activation by SAP-1A requires phosphorylation sites conserved in Elk-1.

The C box exhibits substantial sequence conservation in addition to the conserved S/T-P motifs, including a conserved string of hydrophobic amino acids (see figure 1). Deletion of Elk-1 residues F378 and W379, or their individual substitution by alanine, abolished the ability of NLex.ElkC to regulate transcription in response to serum stimulation (figure 3*b*). The conservation of these and other sequences in the SAP-1 C box suggests that they are also necessary for transcriptional activation, although this remains to be tested directly.

(*d*) *ERK activation is sufficient for transcriptional activation by each TCF C-terminus*

Our previous results with Elk-1 showed that serum stimulation causes phosphorylation of many of the multiple conserved S/T-P motifs that are required for transcriptional activation, and that partially purified ERK2 can phosphorylate Elk-1 at many of these sites

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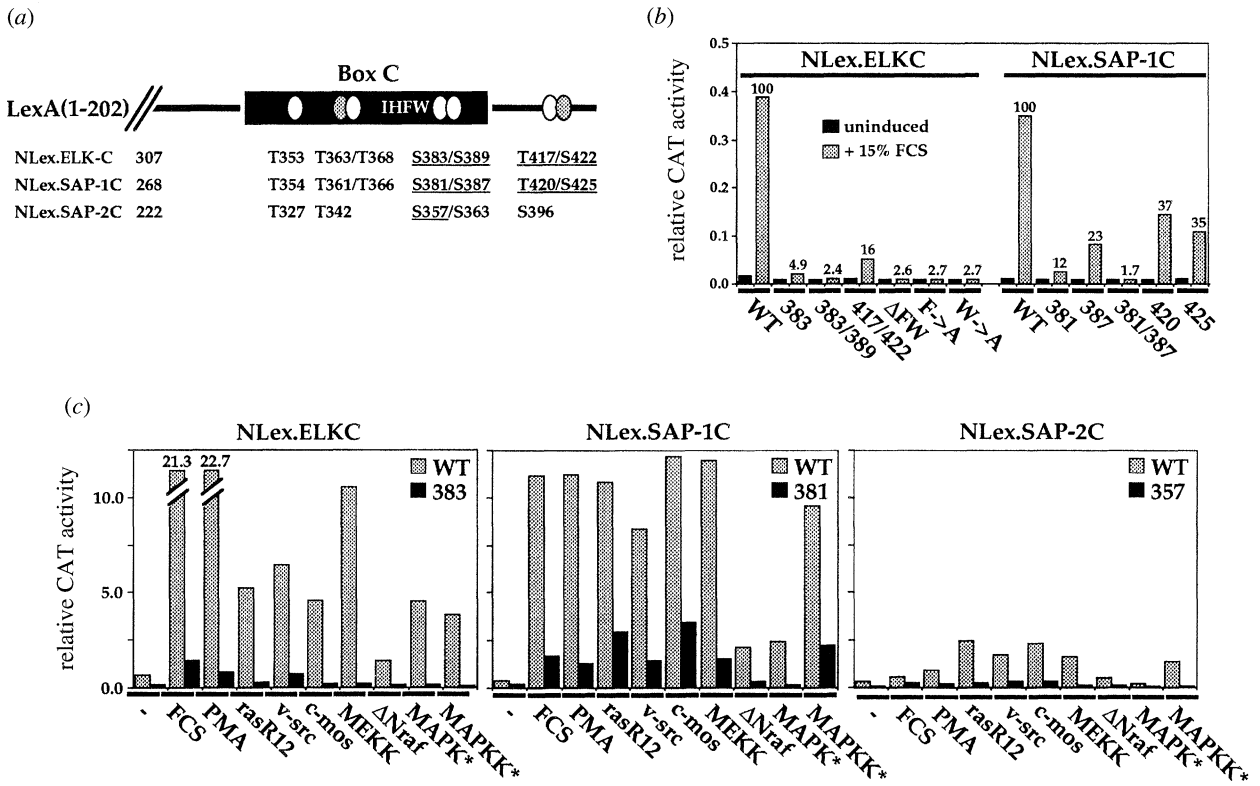

Figure 3. (*a*) Structure of LexA-TCF fusion proteins. TCF homology region C is shown as a solid rectangle. Open or shaded circles show S/T-P motifs present in all (open) or only two (shaded) TCFs. (*b*) Transcriptional activation by SAP-1 requires conserved C terminal S/T-P motifs. NIH3T3 cells were transfected with the reporter gene 2LexOP.tkCAT together with expression plasmids producing the various LexA-TCF fusion proteins, and CAT activity determined in extracts from serum-deprived (solid bars) or -stimulated (shaded bars) cells. A representative experiment is shown; numbers above the bars indicate the average determined from several independent experiments. (*c*) ERK activation is sufficient to activate each of the TCFs. NIH3T3 cells were transfected with the reporter 2LexOP.tkCAT and expression plasmids producing LexA fusion proteins as indicated. Cells were either left untreated, stimulated extracellularly with serum or TPA, or stimulated intracellularly by coexpression of the signalling molecules Ras.R12, mos, v-src, MEKK, ΔN-Raf , ERK2(D319N) (MAPK*), or MAPKK217E/221E (MAPKK*) as indicated. A representative experiment is shown.

in vitro (Marais *et al.* 1993). We have obtained similar results with SAP-1A and SAP-2 (data not shown; see Price *et al.* 1995). To determine whether ERK activation is sufficient for activation of the different TCF C-termini *in vivo*, we tested whether constitutively active signalling molecules that can cause ERK activation, could also potentiate transcriptional activation by the different LexA-TCF fusion proteins. We tested the raf derivative, *Δ*Nraf, and also c-mos, MEKK, and v-src. In addition, we directly activated the ERKs by expression of a constitutively activated MAPKK derivative, MAPKK(217E/221E) (Cowley *et al.* 1994) and the ERK2 mutant, ERK2(D319N), whose Drosophila counterpart is weakly activated by background signal in the ras/raf/ERK pathway in the absence of receptor activation (E. Hafen, personal communication). All the activators tested stimulated transcriptional activation by the Elk-1, SAP-1 and SAP2 C-termini in the absence of growth factor stimulation. Most importantly, activation was efficiently achieved by expression of MAPKK(217E/221E), and even expression of ERK2(D319N) allowed activation, albeit weakly (figure 3*c*). In each case activation was substantially reduced by mutation of the residue corresponding to Elk-1 residue 383 (figure 3C, compare open and shaded bars); a more comprehensive survey of the Elk-1 and SAP-1A mutants showed that activation requires the same S/T-P motifs as are required for growth factor regulation (data not shown). These results show that activation of the ERKs alone can be sufficient for activation of the different TCF C termini.

(*e*) *SRE activation by Rho-family GTPases does not require TCF*

The results presented so far establish the three different TCF proteins as targets for the Ras-Raf-ERK pathway. However, although whole serum, LPA or AlF_4^- ion each activate this pathway in NIH3T3 cells, they do not require TCF to activate the c-fos promoter (Hill & Treisman 1995*b*). We therefore sought to identify signalling molecules involved in activation of the putative additional distinct signalling pathway that targets SRF. Previous studies have shown that at least some of the effects of LPA arise via its activation of RhoA, a small GTPase that defines a subfamily of Ras-related GTPases (for reviews, see Jalink *et al.* 1994) and so we investigated whether constitutively

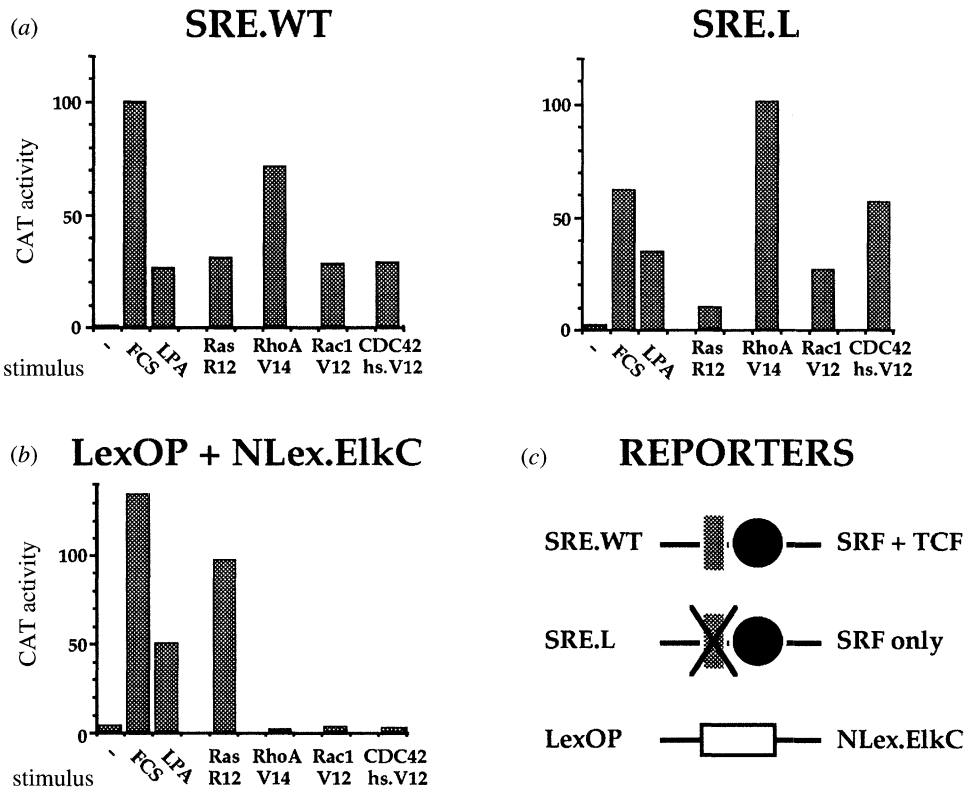

Figure 4. Rho family proteins activate the SRE independently of TCF. (*a*) Activation of the SRE by small GTPases. Cells were transfected with TKCAT reporter plasmids controlled by the wild type SRE (left panel); or SRE.L, which lacks a TCF binding site (right panel), together with expression plasmids producing the Ras.R12, RhoA.V14, Rac1.V12 or CDC42hs.V12 proteins. (*b*) Activation of TCF Elk-1 by small GTPases. Cells were transfected with the Lex2TKCAT reporter and NLexA.ElkC expression plasmids, together with GTPase expression plasmids as above. Data shown in parts A and B are from a representative experiment. (*c*) Reporter plasmid control sequences. Sequences are shown schematically together with their cognate transcription factors.

activated forms of three Rho-family proteins, RhoA, Rac1 and CDC42hs could activate the SRE independently of TCF.

NIH3T3 cells were transfected with CAT reporter genes controlled by either two copies of the intact c-fos SRE, or its derivative SRE.L, which cannot bind TCF (Hill *et al.* 1993) together with expression plasmids producing the activated small GTPases Ras.R12, RhoA.V14, Rac1.V12 or CDC42hs.V12. Coexpression of each of the proteins activated the wild type SRE in the absence of growth factor stimulation (figure 4*a*, left panel). In contrast, the SRE.L reporter responded only weakly to RasR12 but consistently exhibited a slightly enhanced response to the Rho-family proteins (figure 4*a*, compare left and right panels). Activation of SRE.L both by Rho-family proteins and LPA was abolished by a mutation of the SRE that prevents SRF binding (data not shown). To test whether Rho-family GTPases can also activate TCF, we tested their ability to potentiate transcriptional activation by Elk-1 using the NLexElkC reporter gene system described above. Although Ras.R12 strongly activated NLexElkC, coexpression of the Rho-family proteins did not increase activation above background levels (figure 3*b*). Taken together, these data demonstrate that activated Rho-family GTPases can induce TCF-independent SRE activation, and cannot activate the TCFs. In contrast, activated Ras can activate TCF, but activates the SRF-linked signal pathway only weakly. As expected, LPA, which can activate both Ras- and Rho-dependent signalling pathways, efficiently potentiates transcriptional activation by both TCF- and SRF- linked signalling pathways (figure 3*b*).

(*f*) *TCF- and SRF-linked signalling pathways activate the SRE synergistically*

We next investigated how the presence of TCF affects the response of the SRE to Ras- and Rho-linked signal pathways. To do this, we exploited our ability to activate these pathways intracellularly, varying the level of TCF present at SRE.L by use of an expression plasmid encoding an altered-specificity derivative of Elk-1, NLElk, which can bind SRE.L (Hill *et al.* 1993; Hill *et al.* 1994). As a control, we used NL.Elk307Δ, an NL.Elk derivative that can bind SRE.L, but which lacks the C-terminal activation domain (Hill *et al.* 1993; Marais *et al.* 1993; Hill *et al.* 1994). To activate TCF- and SRF-linked signalling pathways independently we expressed either an activated raf derivative, ΔNraf, or the activated Rho-family proteins RhoA.V14 or CDC42hs.V12.

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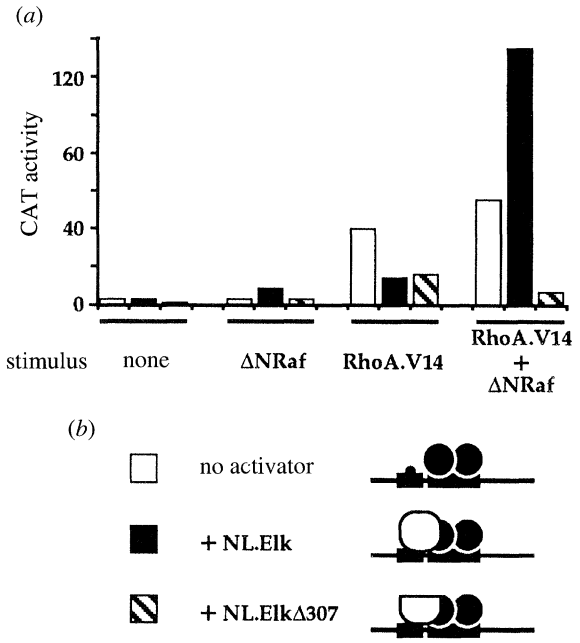

Figure 5. (*a*) TCF- and SRF-mediated signals act synergistically at the SRE. Cells were transfected with the SRE.L-controlled TKCAT reporter plasmid, which lacks a TCF binding site, together with expression plasmids producing the Elk-1 derivatives NL.Elk or NL.Elk307Δ. SRF-mediated signalling pathways were specifically activated by expression of RhoA.V14, and TCF-mediated signalling pathways by expression of ΔNRaf. CAT activity was determined in serum-deprived cell extracts. (*b*) Proteins binding the SRE. The SRE is shown with SRF binding site indicated by a rectangle and the mutated TCF binding site by a nondescript shape. SRF is shown as a pair of circles. NL.Elk, is shown as a rounded square, which is truncated to indicate the defective activation domain of NL.Elk307Δ.

As shown above, activity of SRE.L, which cannot bind endogenous TCF, was strongly stimulated by co-expression of activated RhoA, but not by ΔNRaf; ΔNRaf did not significantly potentiate activation by RhoA (figure 5*a*). Cotransfection of the NL.Elk expression plasmid allowed a weak response of SRE.L to ΔNRaf, but strongly inhibited its response to RhoAV14; however, in combination ΔNRaf and RhoA.V14 synergistically activated SRE.L (figure 4*a*). Synergistic activation required the Elk-1 activation domain since it was not observed upon transfection of NL.Elk307Δ (figure 4*c*, white bars). Similar results were obtained when CDC42hs.V12 and TPA were used to activated the SRF- and TCF-linked signalling pathways respectively (data not shown). We conclude that maximal activation of the ternary complex requries activation of both SRF- and TCF-linked signalling pathways.

4. DISCUSSION

Here we have analysed how different signalling pathways interact with transcription factors bound to the c-fos SRE. We found that the three members of the TCF family of SRF accessory proteins are all targets for the Ras-Raf-ERK pathway, and that TCF binding is necessary for efficient response of the SRE to activation of this pathway. In contrast, serum, LPA and AlF_4^- all activate a novel pathway that can activate the SRE independently of TCF binding. Activated members of the Rho family of ras-related GTPases can activate this pathway, but not the TCFs. Maximal transcriptional activation by the ternary complex requires simultaneous activation of both pathways.

(*a*) *Functional analysis of the three TCFs*

While SAP-1A and Elk-1 form ternary complexes at the c-fos SRE with comparable efficiency both *in vitro* or *in vivo*, ternary complex formation by SAP-2 is inhibited by its C terminal sequences. However a truncated SAP-2 derivative comprising only its A and B boxes binds as well as corresponding truncated derivatives of SAP-1A and Elk-1. Inhibition of DNA binding does not depend on its specific interaction with SAP-2 N-terminal sequences. Although we found that SAP-2 and its mouse homolog ERP/NET behave similarly in our experiments, others have reported that expression of ERP/NET antisense RNA increases transcriptional activity of the c-fos SRE (Giovane *et al.* 1994). However it remains unclear whether the latter effect is a direct one, as reporters lacking a TCF binding site were not examined.

We envisage two kinds of explanation for the apparently anomalous properties of SAP-2. One possible explanation is that it requires modifications of the SAP-2 inhibitory region that we have not reproduced in our experiments. Indeed, MAP kinase-regulated ternary complex formation by the Elk-1 protein can be observed under certain binding conditions (Gille *et al.* 1995*a*). However, our results indicate that if this does occur it must depend on signalling pathways distinct from those activated by serum or v-ras: although these stimuli cause modification of the SAP-2 C terminus, they do not increase ternary complex formation by SAP-2. An alternative view is that SAP-2 may recognise different target sites from the other TCFs. However, like Elk-1 and SAP-1, a SAP-2 truncation mutant comprising its A and B boxes can form ternary complexes on probes containing SRF and Ets binding motifs in a variety of spacings and orientations. Perhaps SAP-2 must interact with other proteins in order to bind DNA efficiently. Future experiments will investigate these possibilities.

(*b*) *Transcriptional activation by the TCF C-terminal region*

The Elk-1 C-terminus contains a transcriptional activation domain, whose activity is potentiated by phosphorylation of multiple conserved S/T-P motifs (Hill *et al.* 1993; Janknecht *et al.* 1993; Marais *et al.* 1993; Kortenjann *et al.* 1994). The C-terminus of each TCF protein can activate transcription (Marais *et al.* 1993). However, for reasons we do not yet understand, transcriptional activation by the SAP-2 C-terminal

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

region is relatively weak and is brought about by activated intracellular signalling molecules but not by serum or TPA stimulation, even though these agents all bring about its phosphorylation. We used site directed mutagenesis to examine the role of conserved potential MAP kinase sites in transcriptional activation by SAP-1A and SAP-2. Alanine substitutions at SAP-1A residues 381, 387, 420 and 425, and at SAP-2 residue 357, reduce transcriptional activation. The data are consistent with the notion that transcriptional activation by the SAP-1A and SAP-2 C-termini is potentiated by phosphorylation at these sites. In addition to the conserved S/T-P motifs, mutations at the conserved Elk-1 hydrophobic amino acids F378 and W379 also leads to an almost complete loss of transcriptional activation. These mutations do not prevent extensive phosphorylation of the C box, and it is therefore likely that they alter a conserved structure involved in activation.

(*c*) *ERK activation is sufficient for transcription activation by TCF C-termini*

Previous studies have demonstrated that activation of the Ras/ERK pathway correlates with transcriptional activation by Elk-1 (Janknecht *et al.* 1993; Marais *et al.* 1993; Kortenjann *et al.* 1994) and that ERK2 is necessary for TCF-dependent activation of the c-fos promoter *in vivo* (Kortenjann *et al.* 1994). We used a variety of activated derivatives of intracellular signalling molecules to test whether the TCFs differ in their ability to respond to ERK activation *in vivo*. The different TCFs behaved identically in this assay: in particular an activated form of ERK kinase, MAPKK(217E/221E) (Cowley *et al.* 1994) efficiently potentiated transcriptional activation. We also found that ERK2(D319N), an ERK2 derivative which is weakly responsive to stimulus-independent noise in the Ras/Raf/ERK pathway (E. Hafen, personal communication) is sufficient to activate each TCF, albeit weakly. Taken together with our demonstration that the C-termini of each of the TCFs can be phosphorylated *in vitro* by partially purified ERK2, these results indicate ERK2 activation is sufficient to potentiate transcriptional activation by each of the TCF C-termini.

Is the ERK pathway the only MAP kinase pathway that can regulate TCF activity? At least two other MAP kinase pathways are present in mammalian cells, controlling activity of the JNK/SAPKs and p38/RK (for references see Han *et al.* 1994; Rouse *et al.* 1994; Sanchez *et al.* 1994; Yan *et al.* 1994; Derijard *et al.* 1995). We found that MEKK and v-src efficiently activate the TCFs; since both of these proteins are implicated in regulation of the JNK/SAPK pathway in addition to the ERKs, it is possible that the JNK/SAPKs may be involved in activation of the TCFs by extracellular stimuli, including stresses such as osmotic shock and UV-C irradation, that normally activate the JNK/SAPKs. Consistent with this idea, both we and others have observed *in vitro* phosphorylation of the TCFs by these MAP kinases (M. A. Price & R. Treisman, unpublished observations; Gille *et al.* 1995*b*; Whitmarsh *et al.* 1995). It remains to be seen to what extent the response to extracellular inducers of these pathways is independent of the ERKs.

(*d*) *TCF independent SRE activation by Rho-family proteins*

We previously demonstrated that serum, LPA, and AlF_4^- ion can activate the c-fos SRE in the absence of TCF (Hill & Treisman 1995*b*). Mutationally activated forms of both RhoA and the Rho-family proteins Rac1 and CDC42hs also activate the SRE independently of TCF. In contrast with Ras itself, the Rho-family GTPases neither activate the TCFs nor the ERKs in transient transfection assays. We show elsewhere that functional RhoA is required for the activation of the SRE by serum, LPA, and AlF_4^- ion (Hill *et al.* 1995). Thus, two kinds of signalling pathway must converge at the SRE, one of which one links TCF activity to the ras-induced ERK activation, while the other links SRF activity to Rho-family induced signals (figure 6). The roles of rac1 and CDC42hs in TCF-independent signalling to the SRE remain to be elucidated. We previously proposed that the target for serum-induced signal pathway was an SRF accessory protein distinct from TCF, that only interacts with the DNA-bound SRF DNA binding domain (Hill *et al.* 1994). Our data establish this putative factor as a potential substrate for Rho-family mediated signals, and we are currently developing methods for its identification.

As yet we have been unable to correlate activation of a particular kinase with activation of the SRF-linked signal pathway. ERK2 activation appears neither necessary nor sufficient: TPA activates ERK2 but not the SRF-linked pathway, and conversely, pertussis toxin blocks LPA-induced ERK2 activation and impairs that by serum without affecting activation of SRF (Hill *et al.* 1995). Similiarly, activation of the JNK/SAPKs and MPK2/p38 is strongly induced by UV irradiation which do not activate the SRF-linked signalling pathways. Thus, although recent studies show that the JNK/SAPKs can be activated by both G protein coupled receptors and a Rac1-mediated signalling pathway (Coso *et al.* 1995; Minden *et al.* 1995) this is not sufficient for activation of SRF.

How, then, are rho-family mediated signals transmitted to the SRE? By analogy with yeast signal transduction systems, we favour the idea that these proteins, like Ras itself, interact with effector kinases that control the activity of MAP kinase cascades. In yeast, both CDC42 and Rho1 are implicated in such interactions, with the kinases STE20 and PKC1 respectively (Simon *et al.* 1995; Zhao *et al.* 1995). According to this model Rho-, CDC42hs and Rac1 mediated signal pathways to SRF would converge at some point downstream of Rho, via a common signalling molecule further downstream. We are currently testing whether mammalian homologs of these kinases such as p65PAK (Manser *et al.* 1994) are involved in Rho-family mediated signal transduction to the SRE.

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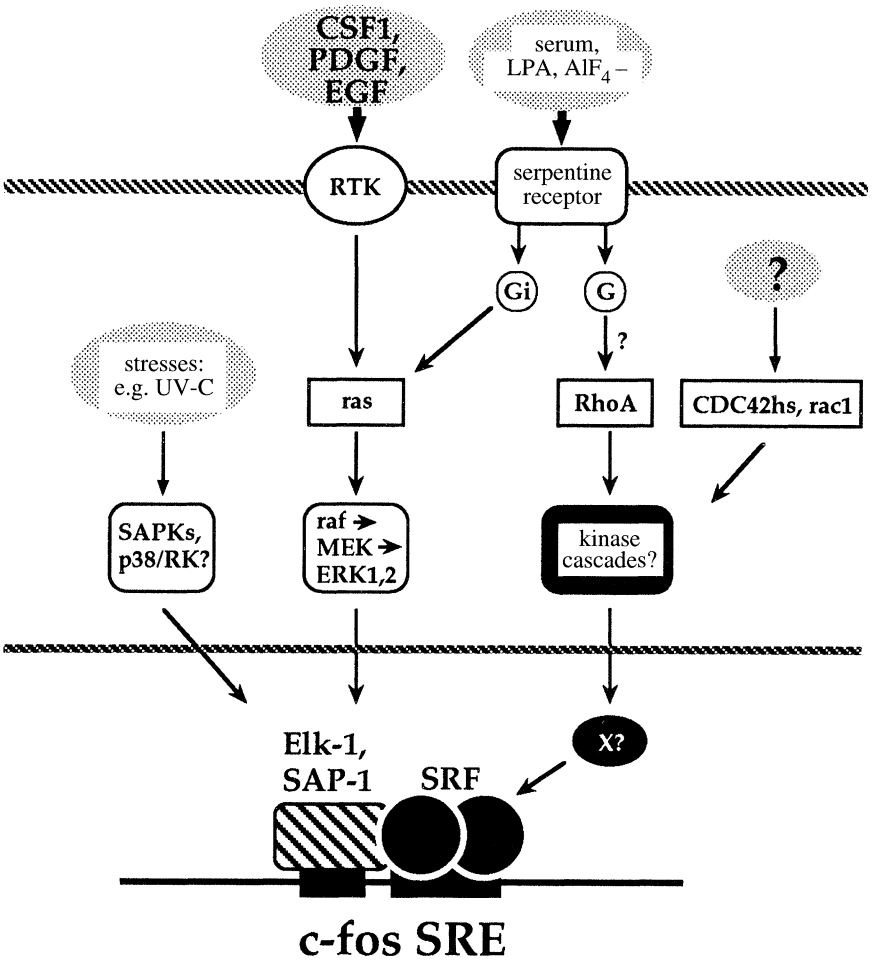

Figure 6. Signalling pathways to the c-fos SRE. (*a*) Signalling pathways to the c-fos SRE (*b*), compared with the *S. Cerevisiae* pheromone signalling pathway to MCM1 (B). Rectangles, ras-superfamily proteins; rounded squares, MAP kinase cascades. For discussion see text.

(*e*) *Interactions between signalling pathways at the SRE*

Our data show that both TCF-linked and SRF-linked signalling pathways cooperate in activation of the c-fos SRE. Although TCF binding is not required for SRE activation in response to activated Rho-family proteins, the presence of inactive TCF at the SRE interferes with Rho-induced activation. However, when both TCF and SRF are simultaneously activated, by stimulation with activated raf and Rho-family proteins respectively, SRE activation is much more efficient than with either stimulus alone. Thus, at SREs at which the ternary complex forms, activation of both SRF- and TCF-linked signal pathways will be necessary for efficient gene activation. This may explain the otherwise puzzling observation that although c-fos promoter mutants that cannot bind TCF remain serum-inducible, their activity can be partially blocked by expression of transcriptionally inactive TCF mutant that can bind the SRE yet cannot be activated in response to signals (Hill *et al.* 1994). It is therefore possible that agents that trigger only SRF-linked signalling pathways will induce only a subset of SRE-containing genes.

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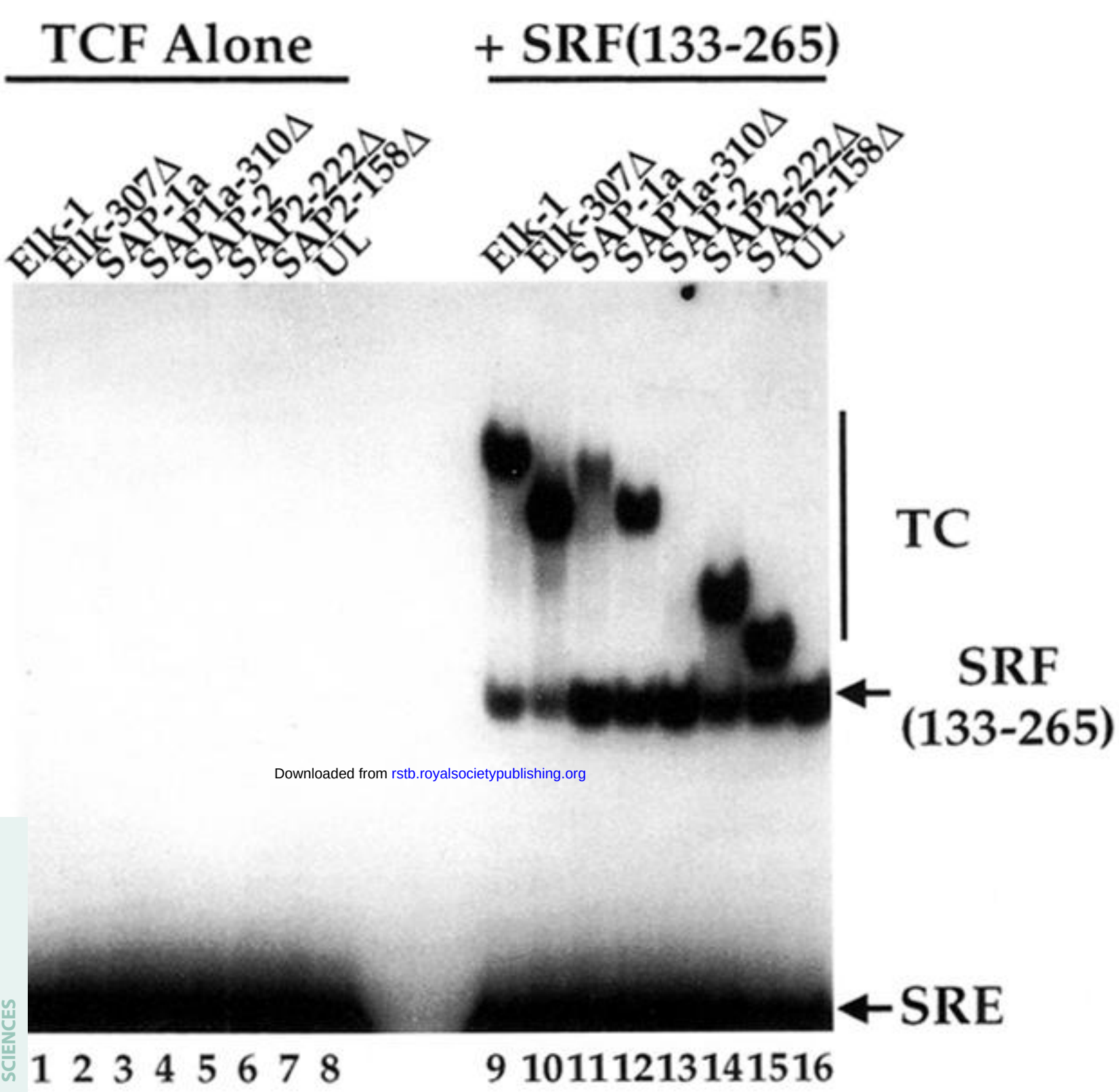
(a)



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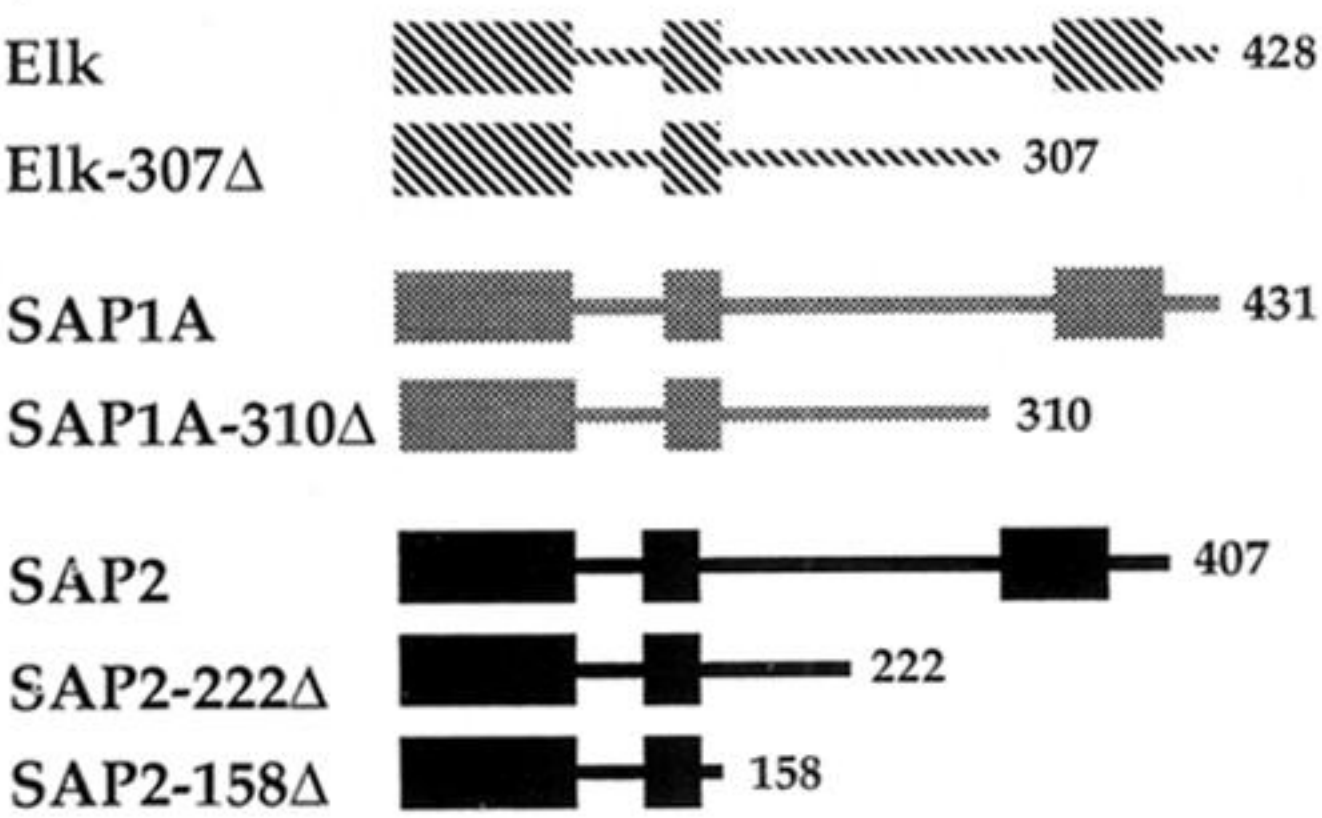

gure 1. Structure and *in vitro* binding by TCF proteins. ernary complex formation *in vitro* by the three TCF oteins. Gel mobility shift assays were performed with a c- SRE probe in the absence (lanes 1–8) or presence (lanes 16) of SRF(133–265), using TCF derivatives produced by *vitro* translation. UL, unprogrammed reticulocyte lysate. ernary complexes, the SRF(133–265):SRE complex, and e probe are indicated. The structure of the TCF derivatives shown below, with the homology regions (Dalton & eisman 1992) shown as boxes.